BIOELECTROCHEMISTRY, THE LIVING STATE, AND ELECTRONIC CONDUCTION IN PROTEINS

Ronald Pethig* and Albert Szent-Györgyi

Laboratory of the National Foundation for Cancer Research
Marine Biological Laboratory
Woods Hole, Massachusetts 02543

The biological importance of charge-transfer reactions involving structural proteins is discussed. Particular emphasis is placed on those reactions which result in the generation of delocalized electronic charges in the protein molecules, and several experimental studies on various protein-methylglyoxal complexes are described which provide strong evidence for the existence of such a process. Such studies have led to the synthesis of a new compound that exhibits interesting physiological activity.

* Permanent Address: Laboratory of the National Foundation for Cancer Research,
University College of North Wales,
Bangor, Gwynedd, U.K. LL57, 1UT.

INTRODUCTION

We divide our surrounding world into the animate and inanimate. This division is normally sharp and unequivocal, which indicates that there must exist some basic physical difference between the two states. It also suggests to us that we should not restrict ourselves to classifying matter solely in terms of the gaseous, liquid and solid state but should also include the living state. The subtle reactivity and sensitivity of living systems is what distinguishes the animate from the inanimate, and because this difference is so sharp it is reasonable to suppose that it has as its basis a specific physiochemical state which should be able to be described in terms of exact science. To ask the question "What is Life?" is relatively meaningless, but we can enquire about the precise nature of the living state and expect to be able to derive scientifically precise answers.

The history of the biological sciences can be traced from the Renaissance period of macroscopic biology which recognized that living systems are built up of organs, to the microscopic anatomists of the last century who showed these organs to be composed of cells, and thence to our modern molecular biology which has achieved so many brilliant successes. Molecules can be decomposed into atoms, and atoms into electrons and elementary particles. Should biology follow physics and enter this sub-molecular world, and if so how far should it follow? To help answer this question we need only remind ourselves of some of the sensitivities that characterize the living state, as for example the ability of a moth to detect single pheromone molecules, the use of acoustic holographic radar by bats, and the sensory power of finger tips. Nature has somehow enabled some snakes to detect ambient temperature changes as small as 10^{-3} ^{o}C, some fishes to respond to electric fields as low as 10^{-6} Vm^{-1} and certain bacteria, bees and birds to use magnetic fields of $5x10^{-5}$ tesla for orientation purposes. Coupled with such sensitivities we also have the subtleties of vision, thought and memory. It would seem reasonable, if not imperative, to consider that some of these sensitivities and subtleties have as their basis mechanisms that operate at the submolecular, electronic, level. Living systems just cannot rely solely for their functions on effects that result from classical mass action interactions involving clumsy macromolecules. The differences between the normal and pathological, between a normal and cancerous cell for example, arise from a variety of very subtle causes. To fully understand the cancer problem, biology must encompass the submolecular sciences and investigate the electronic properties of biological systems. Such studies will naturally fall under the umbrella of bioelectrochemistry. It is very unlikely that subnuclear effects will require attention since the energies involved are far too enormous to be of biological relevance.

Support for the opinion that bioelectrochemistry can lead us towards an understanding of the basic processes that control the living state can be found in the original proposal for this Symposium that was submitted to the Australian Department of Science and Environment and the United States National Science Foundation. In this proposal mention was made of the exciting contributions that bioelectrochemistry has already made in such topics as signal transmission along axons and across synapses, neuromuscular function, electron transfer and energy conversion in cells, antigen-antibody reactions, bone healing and growth, acupuncture and pathogen inactivation. In this selection of topics, which by no means exhausts those that were or could have been cited, charge-transfer interactions at interfaces and the mobility of electrical charges are of fundamental relevance. Broadly speaking, biological charge transport processes can be divided into those that take place in aqueous or lipid environments outside macromolecular complexes and those that occur within such complexes. We are particularly interested in intramolecular charge mobility effects in proteins, and concern ourselves with the transfer and delocalization of electrons and electron "holes" and not with effects associated with the diffusion of protons and other ions within or at the surface of protein structures. The proteins of most relevance to our studies are the structural proteins. The primitive life-forms relied on the catalytic activity of proteins to perform such simple tasks as the making and breaking of chemical bonds which enabled them, for example, to derive energy from foodstuff molecules through the process of fermentation. These simple "vegetative" functions were performed by soluble proteins. The more complex functions and the cellular differentiations that characterize the higher life-forms could only have arisen from proteins possessing a higher degree of reactivity and from the integration of such protein molecules into complex insoluble structures.

The basic philosophy that has led to this emphasis of submolecular biology, its relevance to cancer research, and descriptions of the dielectrics and electronic properties of biological material form the subject matter of several books[1-5]. The object of this present contribution is to outline the progress that we and our colleagues have made to demonstrate that protein molecules are capable of taking part in charge-transfer interactions which result in the appearance of mobile electronic charges within the protein structure. In particular we wish to emphasize the concept that as a result of charge-transfer reactions with other molecules the ground-state energy levels of a protein may become partially desaturated of electron charge. If a well-defined valence band of extended energy states and an associated "tail" of localized states exists for the protein molecule, then the electron "holes" formed by such charge-transfer reactions will be capable of extensive delocalization within the ground-state energy levels of the protein structure. Such a process lends to proteins a submolecular

subtlety of behavior not possible for their electron-saturated counterparts, and we believe that this represents one of the essential evolutionary steps that were required in developing living systems. The molecule which appears to be particularly capable of producing such a charge-transfer reaction with proteins is methylglyoxal.

Energy Bands in Proteins

Proteins represent one of the classes of condensed matter and as such we have no basic reason to doubt that they will exhibit some of the solid-state electronic properties known to occur in conventional amorphous materials and organic polymers. Condensed matter consists essentially of a cloud of electrons held together by nuclei, and it is most often the case even for amorphous materials that the electron energy levels are not randomly distributed in their energy levels but are grouped together into "bands" of allowed energies. We are able to see through a glass window because the valence electron states and the excited electron states of the atoms forming the amorphous glass structure fall into two distinct bands of energy levels separated by at least 3 eV. Discrete electron states do exist in the gap between the energy bands but their number, and hence the extent of electronic excitations in the visible range of energies is much less than if these states were randomly distributed instead of being grouped together into bands. The formation of energy bands is greatly facilitated if the constituent atoms of the material are largely similar in nature and if the atomic lattice possesses some form of regularity. Both of these conditions are present to some extent in proteins.

Proteins are composed of polypeptide chains as shown schematically in Figure 1. Along the polypeptide chain we therefore have a repeating -C-C-N- atomic structure. Each peptide unit has a planar structure since it consists of a delocalized system of π-electrons associated with the π-orbitals of the C and O atoms

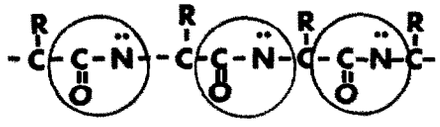

Fig. 1. Schematic representation of a polypeptide chain to show the regularity of the (circled) planar peptide units. For only two of the 20 or so amino-acid residues that can comprise such a chain, the first atom in the side-chain R is <u>not</u> a carbon atom.

together with the lone electron-pair orbital of the N atom, and this π-electron resonance structure is sufficient to produce a significant diamagnetic anisotropy in protein structures.[6]

Of the 20 or so amino-acid residues that comprise polypeptide chains, it is only for the case of glycine and proline that the first atom in the side-chain R is not a carbon atom. We can see therefore that the atomic structure of proteins does possess features of regularity that can assist in the formation of energy bands, and this will be particularly the case for extended structures such as α-helices and β-pleated sheets. The extent to which free charges are able to freely migrate through proteins will depend on the form of the energy bands. If broad bands of extended electronic states exist for the valency ground states and the excited states then coherent wave-like electronic motion is possible in these two bands (referred to as the valence and conduction band respectively). If, however, such bands of extended states are narrow in energy extent (say less than around 2kT), or if only bands of localized energy states exist, then rapid coherent charge transport is not possible and instead the charge motion takes the form of a slow activated hopping or tunnelling process.

Many energy band calculations have been made for model protein structures, beginning in 1947 by Gergely and Evans who based their calculations on the hydrogen-bonded, pseudo-conjugated, network,

$$-C{=}O \cdots H{-}N{-}C{=}O \cdots H{-}N-$$

which runs perpendicular to the main polypeptide chains in the α-helix and β-pleated protein structures. The development of such energy band calculations to the present time has been outlined in reference 5. It will be sufficient for our purposes here to relate that two important points arise from these calculations. Firstly, unlike the early studies, the latest considerations by such workers as Suhai, Ladik and Petrov indicate that the energy band widths are greater for atomic interactions along the polypeptide backbone chains (-C-C-N-C-C-N-) than those resulting from interactions through the hydrogen-bonded networks. Furthermore, the widths of these bands are such as to indicate that coherent wave-like charge transport is possible, at least through the idealized protein structures considered in the theoretical calculations. The second important fact is that all of the calculations indicate that proteins in their natural "pure" state are insulators. The valence band of extended states is completely occupied by electrons and the energy gap between these filled levels and the completely empty ones in the conduction band is so large that at physiological temperatures there is a negligible possibility for electrons to be excited from the valence band to the conduction band. Without the appearance of such delocalized electrons in

the conduction band, or of electron "holes" in the valence band, proteins are unable to exhibit electronic conductivity.

In 1941 it was suggested[7] that the wonderful subtlety of biological reactions could not be produced solely by such clumsy macromolecules as proteins, but had to be produced partly by much smaller and more mobile units which could be nothing other than electrons. The proposal was therefore made that proteins may be conductors. By and large this proposition was rejected and remains so largely today. There are mainly two simple reasons for this. The proteins that have been the most thoroughly studied are by necessity the soluble proteins - the ones that can easily be isolated and crystallized into a pure form. As we have seen such pure proteins will indeed be electronic insulators, as has been shown by many experiments, and besides we have already noted that the soluble proteins are not the ones we expect to exhibit electronic conductivity. It is the structural proteins bearing the main biological function in which we are particularly interested, and it is usually the case when isolating the soluble proteins that the insoluble structural proteins that cannot be crystallized are termed "the residue" and are discarded down the drain! To be functional these structural proteins will be complexed with "impurity" molecules, and if as a result of a charge transfer interaction with such an impurity an electron is donated into the empty conduction band or extracted from the otherwise filled valence band, then the protein will exhibit electronic conductivity. Such a process is analogous to the doping of intrinsic elemental semiconductors such as germanium and silicon to make then n- or p-type. It will be shown here that the proposal made in 1941, that somehow Nature has been able to produce conducting proteins, is correct. One of the molecules that appears to be able to do this, to convert insulating proteins into p-type conductors, is methylglyoxal. It will be appropriate therefore at this stage to give a brief outline of methylglyoxal and its possible great biological significance.

Methylglyoxal

The universal electron acceptor of Life is the oxygen molecule. However, oxygen tends to take up pairs of electrons and as such is not suitable on its own to act as an electron acceptor for proteins. If it did so then all of us would simply "burn-up" very rapidly. It is generally the case for charge-transfer interactions that the donor molecule donates at most a single electron to the acceptor molecule. This would represent a strong charge-transfer interaction in which a bi-radical is formed leaving an unpaired electron on both the donor and acceptor molecule. Most charge transfer interactions are weaker than this, so that on average the transfer of only a fraction of an electronic charge takes place. No new chemical bonds are formed, so instead we talk of a charge-transfer

complex being produced. In such a complex the transferred electron oscillates between the donor and acceptor molecule and prefers on average to remain in the vicinity of its parent (the donor) molecule. It is through such charge-transfer interactions that proteins can be imbued with a submolecular subtlety of behaviour, converting them from a "dead" into a "living" macromolecule.

Oxygen (O=O) can be converted from a divalent acceptor by linking its atoms to carbon as C=O instead of linking them to one another. The carbonyl (C=O) group so formed is a very poor electron acceptor, but this can be improved by first linking two C=O groups to form glyoxal (OCHHCO), and then adding a methyl group to form methylglyoxal (CH_3COCHO) as shown in Figure 2. Methylglyoxal possesses simultaneously a highly reactive aldehydic group with which it can "attack" proteins, and its ketonic group has a relatively low-lying energy level with which it can accept electronic charge. (Nature is simple but subtle!) Methylglyoxal can be produced in living tissues in many ways (for example the removal of one water molecule from triose gives the same chemical formula as methylglyoxal) and it has been found[8] to occur naturally in beef liver in a form bound to the structural proteins. It must also play an important role in cell life, because since 1913 the existence has been known[9,10] of a most active and widely spread enzyme called glyoxalase. The function of this enzyme is to transform

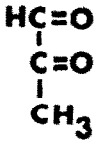

Fig. 2. Methylglyoxal.

methylglyoxal into D-lactic acid, which it does with great speed and efficiency. Nature does not indulge in luxuries, and therefore such an enzyme must have a very important function to perform. However, methylglyoxal together with D-lactic acid are not known to lie on any metabolic pathway, and the purpose of glyoxalase remains a mystery to present biology. This mystery disappears however if the concepts of charge-transfer and electronic desaturation of proteins are taken into account.

If proteins are suspended in the dark in 10 volumes of methanol containing 10% neutralized 40% methylglyoxal solution they assume a stable brown color. The proteins to have been treated in this way have included bovine serum albumin (BSA), casein and lysozyme, which are normally colorless. For the case of collagen the samples are suspended in 0.1 M dichloroacetic acid in the presence of neutralized methylglyoxal, and the addition of methanol results in the precipitation of a brown complex. Scintillation counting measurements by M. Arnold and Joy Behi have been used to determine the extent of binding of C_{14} labeled methylglyoxal to the above mentioned proteins as well as to chymotrypsin, chymotrypsinogen, cytochrome-c and fibrinogen, and acid hydrolysis followed by amino-acid analysis of the methylglyoxal complexes of BSA, and casein in the laboratory of L. Lorand has shown that practically all of the arginine and 80 to 90% of the lysine groups are involved in the reaction.

When the free amino groups of lysine and the terminal polypeptide amino groups are dimethylated, the incubation reaction with methylglyoxal does not produce a brown protein complex having the electronic properties described in the next section, and furthermore with Jane McLaughlin and P.R.C. Gascoyne we have been able to show that the color and electron spin resonance signal intensities exhibited by the complexes are directly related to the number of "unblocked" lysine residues. When the arginine side-chains of BSA are blocked from reacting with methylglyoxal, the protein still reacts with methylglyoxal to produce a stable brown complex exhibiting similar electronic properties to those exhibited by the unmodified BSA complexes with methylglyoxal. These results suggest that the relevant reactions for our studies involves the lysine side-chains and we believe that the first step is the formation of Schiff base (-C=N-) linkages to the ε-amino groups. Otto et al have shown[11] that such a Schiff base is as good an electron acceptor as methylglyoxal itself and they also show that no change of the main polypeptide chain configurations is required for the lysine side-chain to bend back to enable a charge-transfer interaction to occur between the electron-donating "next-door" peptide unit and the electron-accepting Schiff base. It has not escaped our attention that our methylglyoxal-protein complexes may have important parallel similarities to rhodopsin, and to bacteriorhodopsin isolated from the purple membrane of Halobacterium halobium, where retinal is linked to a lysine residue by a Schiff base. The presence of SH groups in the protein is also involved in producing the final brown color of the protein-methylglyoxal complexes, but as yet we do not understand the process involved. This too may be of great interest since sulfhydryl groups are known to play significant roles in many biological reactions.

Physical Measurements

Various physical measurements have been made in our laboratories to investigate the electronic and dielectric properties of the colored protein-methylglyoxal complexes. The main purpose of these studies has been to show that when methylglyoxal is incorporated into the structure of a protein molecule, a charge-transfer interaction occurs resulting in the creation of mobile electron "holes". These measurements will now be outlined, with emphasis being placed on the latest and as yet unpublished work. Precise details of the experimental techniques and analyses can be derived from earlier publications (references 13, 14).

The measurements have been made on dry protein samples, or ones containing at most 35% water, that have been compressed into the form of polycrystalline discs. The question can therefore readily be asked: Can such studies be of any biological relevance? As a first step to investigate the inherent electronic properties of protein-methylglyoxal complexes we believe that such measurements can provide useful data. As mentioned earlier, the latest energy band calculations indicate that electronic delocalization is likely to be most favorable along the polypeptide backbones of protein structures rather than through the hydrogen-bonded structures that help stabilize the protein's tertiary structure. If that conclusion is applicable to proteins generally, it means that denaturation and dehydration of the proteins should not seriously alter the basic electronic properties associated with their primary structures. Also, in this way, the fact that we have tended as a first step to investigate the soluble proteins is not at variance with our long term aim to study the structural proteins since the existence of the polypeptide backbone is common to proteins in general. The possible lack of sufficient hydration may also not be too serious, since in their natural state many proteins are bound into hydrophobic lipid matrices. The energetics of charge separation and motion are largely governed by the relative permittivity (dielectric constant) of the surrounding medium, and in this respect it can be calculated (reference 5, pp 63-66) that the internal structures of proteins have an effective high frequency relative permittivity of the order 2.6, and this is a value which will not be greatly increased on hydration of the protein. The particular electronic properties of a protein in its natural state will obviously depend on its precise conformation, environment, and interactions with other molecules. In these our early studies we can only hope to observe the tendencies of the possible submolecular electronic behaviour of protein structures, but at the same time these tendencies can provide valuable clues as to what future discoveries will reveal.

Steady State Conductivity

Following the earlier observation[14] that under the same atmospheric conditions the d.c. conductivities of casein - methylglyoxal samples were some three orders of magnitude greater than those of uncomplexed control casein samples, we have with S. Bone extended such conductivity measurements to include the study of other proteins. For the case of casein, BSA and lysozyme steady state conductivities σ of the dry methylglyoxal-complexes follow the standard semiconduction equation with the activation energy

$$\sigma = \sigma_o \exp(-\Delta E/kT) \qquad (1)$$

ΔE having a value typically of the order 0.7 eV for temperatures above 250 K and a value around 0.3 eV below 250 K. This behaviour is demonstrated in Figure 3. Of the proteins studied, collagen-methylglyoxal samples have consistently exhibited the largest steady-state conductivity with the largest recorded value being 2×10^{-9} mho/m at 297 K. However, this value is reduced after temperature cycling and good semiconductivity activation plots have been obtained only after the conductivity, measured at room temperature, has fallen by some two orders of magnitude below its initial high value and the Δ E value has stabilized at a value around 1.1 eV. This would suggest that the conductivity of the dry collagen complexes is sensitive to molecular conformational changes. Under experimental conditions similar to those used here, most dry proteins have conductivities of the order 10^{-16} mho/m at room temperature and a Δ E value of the order 1.4 eV. This shows that incorporating methylglyoxal into the structure of proteins greatly increases their electrical conductivity.

Interesting results have been obtained for the protein α-conglutin kindly prepared for us from lupin seeds by Dr. K. Moore of the University of Bath. Measurements by J. Fothergill on various dry preparations of this protein have consistently shown the room temperature conductivity to be of the order 5×10^{-12} mho/m. On complexing with methylglyoxal this conductivity has decreased by two orders of magnitude, which is the opposite effect obtained with the other proteins. The γ-conglutin is thought to possibly contain traces of phenolic material, and it is possible that this forms a charge-transfer complex to donate electrons into the protein structure. The electron-acceptor action of methylglyoxal could then compensate for this effect by either removing these excess electrons or by directly producing a chemical modification of the phenols.

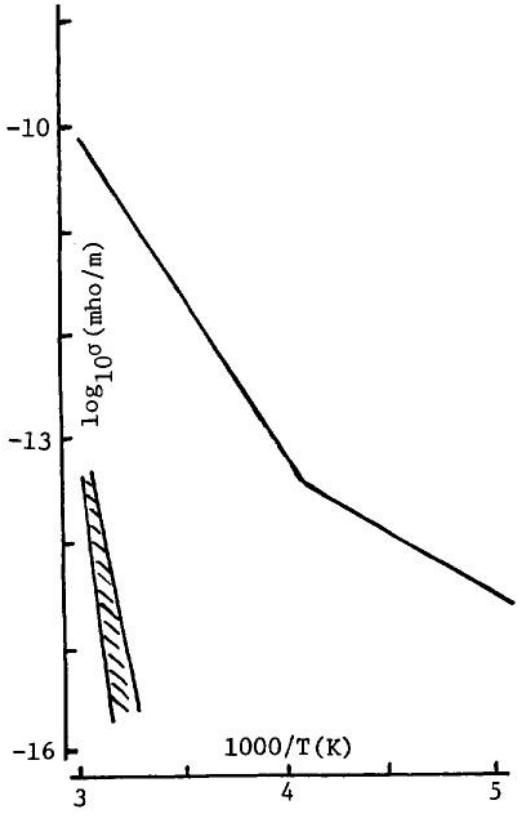

Fig. 3. The typical temperature variation of the dry-state conductivity of proteins that have been complexed with methylglyoxal. Also shown is the range of conductivities normally exhibited by dry untreated proteins.

Electronic Transference Number

The increase in conductivity of the protein-methylglyoxal compared with the normal control proteins could conceivably have arisen through the incorporation of ionic impurities into the samples. The possible contribution of ionic conduction effects has been investigated using a simple electro-chemical technique first described by Liang[15] for the characterization of solid electrolytes. The technique we have used to investigate the protein complexes has been described elsewhere[14] in detail, but a simple description can be obtained from Figure 4.

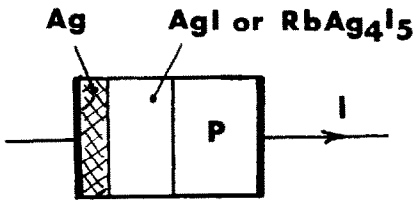

Fig. 4. The configuration used for the determination of electronic transference numbers. The symbol Ag refers to a silver mesh electrode embedded into an ionic conductor (AgI or Rb Ag_4 I_5) and P represents the test protein material. The current I is supplied by a constant current source.

Basically, if a constant electric current is passed from left to right in the arrangement shown in Figure 4, silver will be deposited at the interface between the test protein-complex sample P and the ionic conductor only if electrons are mobile in P. The amount of silver deposited will be proportional to the electronic transference number of the test sample. The measurement procedure consists of first passing a constant "deposition" current through the assembly with the protein sample at the negative potential. The surface of the protein sample in contact with the ionic conductor is then exposed, this surface is scraped to remove any deposited silver and the scrapings are then incorporated into a compressed graphite disc. This disc is then pressed into the ionic conductor material and a constant "oxidizing" current is passed through them of such a polarity that the initial ionic current requires the sacrificial electrode action of the silver scrapings in the graphite. A rapid rise of the voltage monitored across this assembly indicates the endpoint for the oxidation of the silver scrapings since it represents the increased potential required to dissociate other ions in order to maintain the constant oxidizing current. The electronic transference number is given by the ratio of the total oxidizing charge to the original deposition charge.

The results[14] for the dry casein-methylglyoxal complex gave electronic transference number values in the range from 0.75 to 0.89. Values in the range 0.8 to 0.9 have been obtained by J.C. Fothergill for dry BSA and collagen complexes. The technique will tend to underestimate rather than over-estimate the electronic transference number, so that these high values that have been obtained can be taken to indicate that the conductivity of the protein-methylglyoxal samples is not dominated by ionic impurities but instead is predominantly electronic in origin. Values for the conglutin samples have been determined by J.C. Fothergill to be

greater than 0.87, so here again the influence of ionic conduction effects can only be small.

Dielectric Measurements

Measurements of the a.c. conductivity σ (ω) and complex relative permittivity ($\varepsilon_r = \varepsilon' - i\varepsilon''$) have been made over the frequency range 10^{-5} Hz to 33 GHz. As the measurement frequency increases the conductivity increases above the steady state value, and the typical results obtained are shown in Figure 5 for the case of the dry untreated protein and for the dry protein-methylglyoxal complex. The a.c. conductivity of the methylglyoxal-complexes merges into the conductivity characteristic of the untreated proteins so that at micro-wave frequencies the conductivities have similar values to within experimental accuracy. Over a wide frequency range the untreated proteins exhibit a frequency-dependent conductivity of the form

$$\sigma (\omega) = A \omega^{n}$$

with the exponent n being close to unity, and this is a behaviour considered to arise from the presence of dipoles and activated "hopping" charge carriers exhibiting a wide distribution of relaxation times.

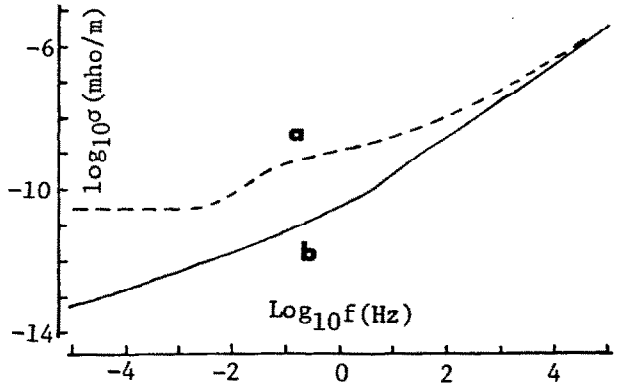

Fig. 5. (a) The typical frequency variation of the conductivity of a dry protein-methylglyoxal sample, and (b) of a dry untreated protein sample at 300 K.

In the frequency range around 10^{-3} to 10^{-1} Hz at room temperature the protein-methylglyoxal complexes exhibit a dielectric dispersion which can be seen as a slight "bump" in the conductivity curve. This dispersion can be shown more clearly as the frequency variation of the dielectric loss factor ε'', as shown in Figure 6 for the dispersion typically observed by S. Bone for the collagen-methylglyoxal complex at room temperature. For each dielectric loss peak the characteristic relaxation time τ is given by

$$\tau = 1/2\pi f_m \tag{1}$$

where f_m is the frequency at which the maximum dielectric loss occurs. With increasing temperature the loss peaks are displaced toward higher frequencies with the total area under the ε'' versus log f plot remaining unchanged within experimental error. From such a temperature variation the dielectric dispersions are found to obey an activated law of the form

$$\tau = \tau_o \exp (W/kT) \tag{2}$$

with the activation energy W having a value very close to that of the conductivity activation energy Δ E of equation (1).

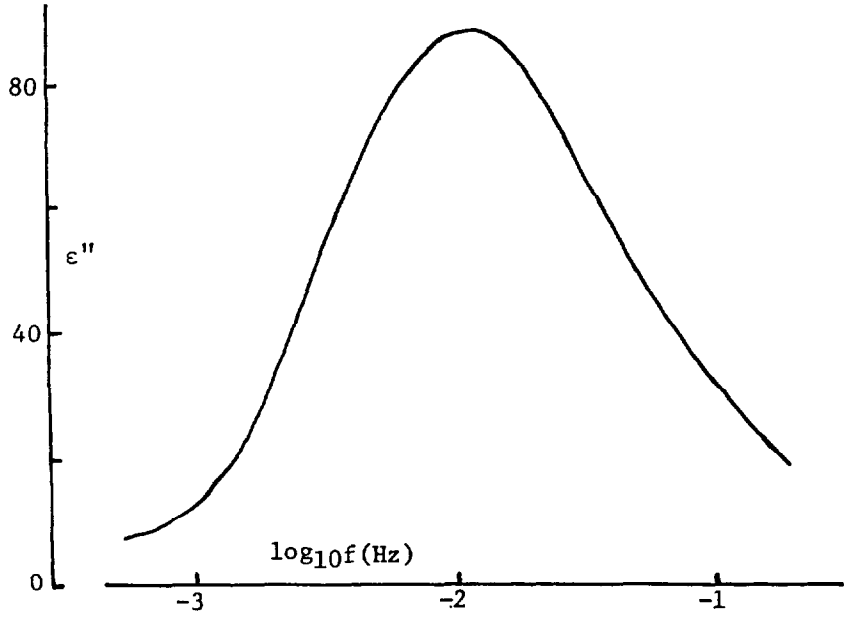

Fig. 6. Dielectric dispersion observed for the collagen-methylglyoxal complex.

A model has recently been proposed[16] to describe the occurrence of such an effect in terms of the activated hopping of charge carriers over potential energy barriers. In this model the long range transport of such hopping carriers controls the steady state conductivity while localized hops give rise to the dielectric dispersion.

Support for the concept that the dielectric dispersion arises from a hopping charge carrier mechanism also results from a close analysis of the dispersions. Firstly, the dispersions are found to be independent of the electrode material used for the test samples, and they remain in existence if thin sheets of PTFE are placed between the electrodes and the test samples. The frequency of maximum dielectric loss, f_m, has also been found to be independent of sample thickness for thicknesses in the range 0.6mm to 6mm. These results show that the dispersions are related to sample bulk effects and not to effects associated with the electrode-sample interface or to the rate at which delocalized charge carriers migrate across the sample. The results of high electric field effects, to be described later, also give supporting evidence for the dispersions being a bulk effect. The main factor that leads to the conclusion regarding the existence of hopping charge carriers is the magnitude of the dielectric dispersions observed, since these dispersions are generally too large to be described in terms of the relaxation of conventional molecular dipoles.

For a set of dipoles of dipole moment μ and concentration Nm^{-3}, then it follows from the theories of Debye and Onsager that

$$2.3\int_{-\infty}^{\infty} \varepsilon'' \, d(\log_{10} f) = \frac{\pi(\varepsilon_s+2)(\varepsilon_\infty+2) N \langle \mu \rangle^2}{54\varepsilon_0 kT} = (\pi/2)(\varepsilon_s-\varepsilon_\infty) \quad (3)$$

where ε_s and ε_∞ are the limiting relative permittivity values at frequencies below and above the dispersion region, respectively, and ε_0 is the permittivity of free space. From an analysis of the dielectric dispersions exhibited by the methylglyoxal-complexes of casein, collagen, BSA and lysozyme and derived values for the product $N \langle \mu \rangle^2$ have typically been of the order $1.2 \pm 0.2 \times 10^{-31}$ Coul^2 m^{-1}. It is worthwhile to remind ourselves here that the normal, uncomplexed, proteins do not exhibit this dielectric dispersion, and so it is reasonable to assume that the presence of the methylglyoxal molecules in the protein structure is responsible for the dispersion. Detailed analyses[12,13] of the dispersions have resulted in the conclusion that the dispersions cannot arise from relaxations of the molecular dipole moment of the methylglyoxal molecules, or for that matter of any other conventional molecular dipole moment, but instead can only be understood in terms of the relaxation of delocalized charge carriers

over potential energy barriers. The generation of these delocalized charges is considered to arise from the charge-transfer interaction between the donor peptide units of the protein and the acceptor Schiff bases that link the methylglyoxal to the lysine side-chains. A similar interpretation in terms of "hopping" charge carriers has been given[17] for the dielectric dispersion that occurs in the perylene-chloranil charge-transfer complex, and a theoretical analysis by Lewis[18] of the dispersions exhibited by the protein-methylglyoxal complexes also gives support to the hopping model.

Finally, the molar entropy of activation ΔS for the activated dielectric dispersion process can be estimated by assuming that τ_o in equation (2) is given according to chemical reaction rate theory by

$$\tau_o = (h/kT) \exp (- \Delta S/k) \qquad (4)$$

At room temperature the derived value for ΔS is of the order of -10 cal per deg. mol, which implies that the relaxation process is associated with some degree of ordering of the protein molecular structure.

High Electric Field Effects

The conductive and dielectric properties of the protein-methylglyoxal complexes have been investigated as a function of the applied electric field stress for values up to 10^7 Volt/m. Apart from the fundamental information they can provide, such high field studies are also of biological relevance. Cells typically have a potential of between 50 to 100 mV across their outer membranes, and as a result the proteins embedded in the membrane will experience a field stress of the order 10^7 Volt/m. Fields even greater than this can occur, as for example those of the order 6×10^7 Volt/m associated with proton gradients across the mitochondrial inner membrane. Most electrical materials scientists would be extremely interested to learn of these materials that function at such high field stresses, and they would be astounded to learn that they do so in association with aqueous electrolytes. Many conventional dielectric materials would suffer electrical breakdown under such conditions. Any study of the electronic and dielectric properties of biological materials should include the high electric field effects, since it will be these properties, rather than the low field ones, that will most likely be of the greatest biological relevance.

When the steady state conductivity data determined at constant temperature are plotted as log (current) against $F^{1/2}$, or log (conductivity) against $F^{1/2}$, where F is the applied electric field strength, straight line plots are obtained only for the log (conductivity) data[13]. This result is consistent with

the sample conductivity being limited by the so-called Poole-Frenkel effect, rather than by Schottky electron emission from the electrodes. The Poole-Frenkel effect is a bulk effect which occurs for materials whose conductivity is limited by the rate at which the charge carriers are thermally assisted over potential energy barriers. With increasing applied electric field stress the effective height of the barriers is reduced and the conductivity increases. In some other materials the electrical current that can pass through them is limited by the rate at which the charge carriers can be injected from the electrodes into the material. This in turn is limited by the potential energy barrier at the electrode-material interface. By increasing the voltage applied across this interface, the surface potential barrier decreases in height and the current through the material increases. Further details of these effects, together with the other information that can be derived from them for the protein-methylglyoxal complexes, are described in an earlier publication[13]. The observation of the Poole-Frenkel effect by itself is of most significance since it helps confirm that the conductivity of the protein-methylglyoxal samples is limited by potential energy barriers in the bulk of the sample.

The magnitude of the dielectric dispersion observed for the protein-methylglyoxal samples has been found to vary significantly with the strength of the electrical field applied to the samples. This is demonstrated in Fig. 7 from which it can be seen that the area under the plot of ε'' against log(f) decreases logarithmically with increasing field strength. This effect cannot be interpreted in terms of the conventional field saturation of dipolar polarizability, but is understandable in terms of a model involving the hopping of charge carriers over potential energy barriers. With increasing applied field stress the frequency f_m of maximum dielectric loss first increases slightly and then falls to a value slightly less than the original low field value of f_m. From equations (2) and (4) this implies that the larger field strengths induce some form of molecular ordering in the samples.

Hydration Studies

The results described so far have been those performed on dry samples. Measurements have also been made as a function of hydration. The amount of water contained in the protein samples has been determined from hydration isotherms obtained using both a conventional weighing balance and the relatively new quartz crystal resonator technique.[19]

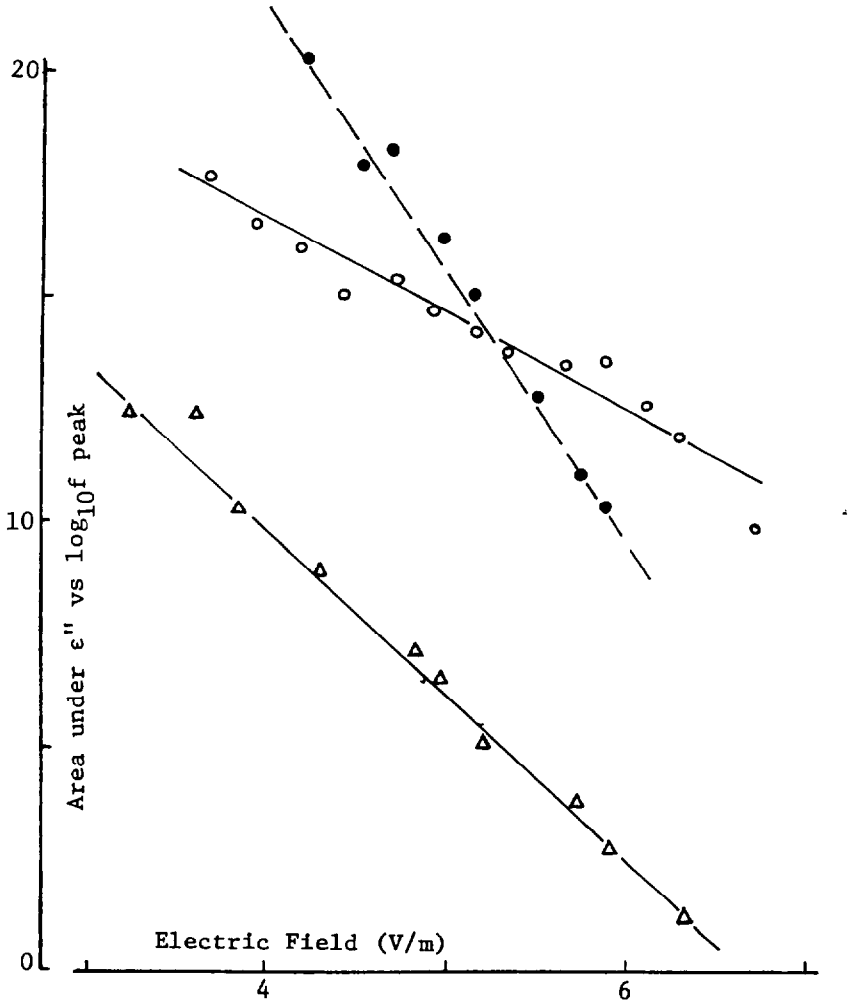

Fig. 7. The variation of the area under the plot of against log(f) as a function of the applied electric field for various protein complexes. • Lysozyme, o Collagen, Δ BSA - (complexes with methylglyoxal).

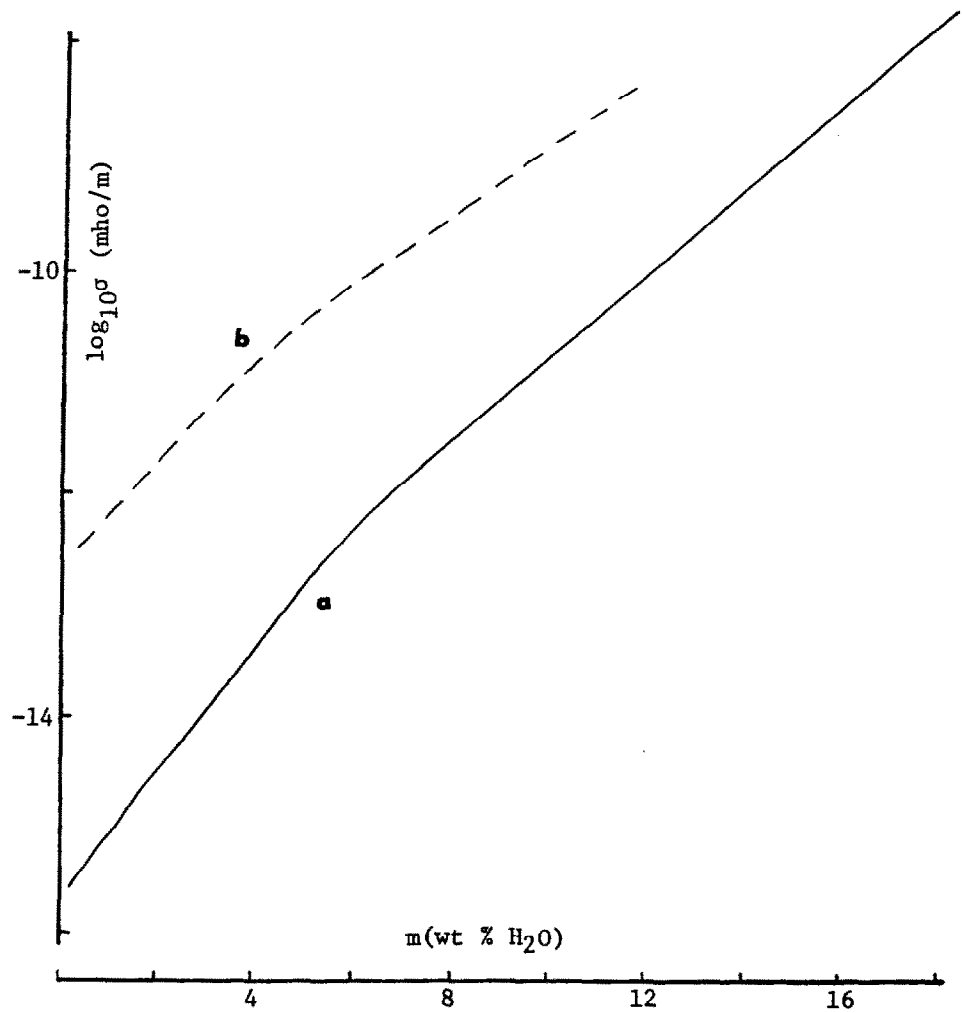

Fig. 8. The typical variation of the steady state conductivity with hydration for (a) untreated proteins and (b) protein-methylglyoxal complexes.

The steady-state conductivity of the protein samples increases rapidly with increasing water content and typical results obtained by Joyce Eden are shown in Figure 8. Over a considerable hydration range the conductivity varies with the amount m (weight percentage) of absorbed water according to the relationship,

$$\sigma(m) = \sigma_D \exp(\alpha m)$$

where σ_D is the dry-state conductivity and α is a constant. At a value of m of around 5wt%, there is a change in this relationship so that $\alpha \simeq 1.3$ for $m < 5$wt%, and $\alpha \simeq 0.0$ for $m > 5$wt%. This effect is considered to be associated with the fact that at $m \simeq 5$wt% nearly all of the protein primary water sorption sites are occupied by water molecules and population of the secondary hydration sites is beginning to occur.

With increasing hydration the frequency f_m of maximum loss of the dielectric dispersions is found to increase, so that the corresponding relaxation time τ varies as

$$\tau = \tau_o \exp(-\beta m)$$

where again at $m \simeq 5$wt% there is a change in the value of the proportionality factor β. Although the frequency f_m changes, the area under the ε'' versus log(f) curve does not change with hydration, indicating that the observed dielectric dispersions are not directly related to the relaxation of water molecule dipoles.

Microwave Hall Effect

Steady-state conduction and low frequency dielectric measurements on biological materials provide details of their macroscopic properties and are unable to give direct information regarding the submolecular electronic properties of the biomolecules. For example, the dielectric dispersions observed for the protein-methylglyoxal samples most likely arise from the relaxation of charge carriers over potential energy barriers that occur either between protein molecules or between crystallites, and the steady-state conductivity is limited by the number of charge carriers that are capable of long range motion over many such barriers. Also, any attempts to determine the charge carrier mobility using steady-state or low frequency measurements will produce a macroscopic mobility μ_m of value given by

$$\mu_m = C\,\mu_o \exp(-E/kT) \qquad (5)$$

where E is the mobility activation energy of value equal to the average height of the potential energy barriers limiting the long range motion of the charge carriers, C is a factor that is determined by the energy distribution of these barriers, and μ_o is the macroscopic mobility. We are particularly interested in obtaining the value for μ_o. Earlier we mentioned that the extent to which free charges will be able to freely migrate through proteins depends on the form of the energy bands associated with their molecular structures. Two extremes of charge carrier mobility were described; one where coherent wave-like motion occurs through a broad band of extended energy states, and the other where the charge carriers slowly diffuse by a process of thermally activated hopping, rather like a form of Brownian motion, via localized states. From several lines of reasoning it can be shown (e.g. ref., 5, pp 265, 266) that the borderline between these two modes of charge transport corresponds to a mobility value of the order 1×10^{-4} m^2/V sec. If it can be demonstrated that the microscopic, submolecular, charge carrier mobility for delocalized holes or electrons in proteins has a value in excess of 1×10^{-4} m^2/V sec then this will provide very strong evidence for the existence of broad bands of extended energy states in their molecular structures.

Mobility values for conventional semiconductors have largely been obtained by measurement of the Hall effect. The samples have mostly taken the form of large single crystals, which in fact represent giant molecules of the test material, so that steady-state and low frequency measurements have been able to provide values for the microscopic (Hall) mobility. Unfortunately, samples of this form are not possible for proteins, and both inter- and intra-molecular effects affect measurements. By increasing the measurement frequency to a high enough value, it is possible to retain delocalized charge carriers within microscopic conduction pathways. The charges simply oscillate back and forth in response to the alternating electric field, having insufficient time to surmount in any one direction the energy barriers that disrupt the conduction pathways. The Soviet physicist E.M. Trukhan was the first to attempt high frequency (9 GHz) Hall effect measurements on biological materials, and such studies were later taken up by Eley and Pethig, and then by Bogomolni and Klein. Measurements at such a high frequency overcome effects associated with inter- and intra-molecular defect barriers, and an added advantage is that the technique does not require the use of electrodes. A summary of these microwave Hall effect studies, together with a description of the measurement technique, has been given elsewhere[20].

With T.E. Cross we have attempted to measure the Hall mobility for the protein-methylglyoxal samples using a 33 GHz Hall measurement apparatus[21]. Of the various samples studied, the collagen-methylglyoxal complex has given the most consistent result. The polarity of the Hall effect is positive, which from conventional energy band theories indicates that the electronic conduction in the collagen complex arises from the transport of electron "holes". The magnitude of the Hall mobility can be estimated to be within the range 1-50 x $10^{-4}m^2/Vsec$, which suggests that a well-defined valence band of extended states exists for the collagen structure. Several problems complicate a precise interpretation of the microwave Hall effect, and there are also theoretical difficulties regarding the magnitude and polarity of the Hall effect exhibited by charge carriers moving via localized states or in narrow energy bands. Hopefully these problems will be resolved, and Hall effect measurements will prove to be a valuable addition to the various techniques that can be used to study the solid-state electronic properties of biomaterials.

Electron Spin Resonance (e.s.r.)

In the charge-transfer reaction with methylglyoxal it is envisaged that electrons are transferred from the polypeptide backbone of the protein to the methylglyoxal acceptor attached to the lysine groups. Such a reaction should lead to the separation of electron pairs, and as such should give rise to an e.s.r. signal. The early measurements[14] for dry samples indicated that the untreated control proteins had a free electron spin density of the order 10^{15} spins/g, whereas the protein-methylglyoxal samples gave the much larger result of around 10^{18} spins/g, which was an increase roughly mirroring the increase in the measured electronic conductivity. The e.s.r. signals for both the normal and methylglyoxal-complexed proteins were centered at g = 2.005, which is sufficiently off the g = 2.0023 value expected for a completely free electron to indicate that the unpaired electrons did in fact possess a small amount of spin orbit coupling. From the variation of the shape of the e.s.r. signal as a function of the microwave power incident on the test samples, it was concluded that the total signal was composed of two separate free radical species having different spin-lattice relaxation times.

These preliminary e.s.r. results have now been confirmed by P.R.C. Gascoyne, as well as in the laboratory of H.M. Swartz. Gascoyne has also extended the measurements to include samples in aqueous solution and finds no discrepancy with the earlier results obtained for the dry samples. Using a computer technique to analyze the hyperfine structure he has identified two components of the e.s.r. signal: one remains constant as a function of hydration and the other component can be understood in terms of

proton splitting. By varying the number of lysine groups of BSA that have been "blocked" through dimethylation, the magnitude of the e.s.r. signal has been found to be related to the number of free lysine groups available to react with the methylglyoxal, and the number of spins/g for the protein-methylglyoxal samples has been found to be constant over the temperature range from 100 to 240 K. Finally, it is of interest to note that the g-value of 2.005 observed for the protein samples is the same as that commonly observed for e.s.r. signals exhibited by biological tissues.

DISCUSSION OF THE PHYSICAL MEASUREMENTS

To summarize these various experiments we can say that when BSA, casein, collagen and lysozyme are reacted with methylglyoxal a brown stable complex is formed. The steady state conductivity of these brown complexes is on the average some three orders of magnitude greater than that exhibited by the normal untreated proteins, and measurements of the Poole-Frenkel effect and electronic transference number indicate that this increase in conductivity is a bulk effect of electronic rather than ionic origin. If the lysine sidechains of the proteins are dimethylated then after the incubation reaction with methylglyoxal the proteins remain white and of very low conductivity. The brown protein-methylglyoxal samples have been found to have a free radical concentration some three orders of magnitude greater than that of the normal untreated proteins and they also exhibit a pronounced low frequency dielectric dispersion not found in normal proteins. These dielectric dispersions can be understood in terms of the hopping of charge carriers over potential energy barriers, and at least for the case of the collagen-complex the Hall effect measurements indicate that these charges are holes that are delocalized in the protein valence band.

The steady-state conductivity, and the frequency at which the dielectric loss peak is a maximum, increase markedly with increasing hydration. The potential energy barriers that limit the conductivity and dielectric relaxation process are most likely to be coulombic in origin, and as such the barrier heights will have an energy profile of the form,

$$F = q^2/4\pi\varepsilon_o\ \varepsilon_r d \qquad (6)$$

where d is the separation distance between counter charges of charge $|q|$. The effect of increasing hydration can therefore be understood in terms of the dipolar water molecules increasing the effective permittivity ε_r of the dielectric medium, and thereby

decreasing the potential energy barrier heights. For the counter charges to be completely free from each other requires F≈kT which for the dry proteins corresponds to the separation distance d being greater than around 18 nm. Such large spacing between the counter charges allows us to think in terms of the macroscopic permittivity ε_r. The counter charges for our scheme would be the electron on the methylglyoxal acceptor and the electron "hole" in the polypeptide backbone.

It seems clear that the lysine side-chains are directly involved in the interaction with methylglyoxal to form the brown protein-complex having an increased electronic activity compared to that of the untreated proteins. The involvement of a Schiff base linkage between the ε-amino group of the lysine side-chain and the methylglyoxal moleceule also seems most probable, but this still remains to be rigorously demonstrated. Another question to be resolved is the exact interpretation to be given to the observed e.s.r. signals. We would like to believe that the signals are directly related to the transfer (unpairing) of charge from the polypeptide backbone to the methylglyoxal acceptor attached to the lysine group. The measured steady-state conductivity then arises from the long-range mobility of one or both of those unpaired charges, and the dielectric dispersion is associated with their localized motions. At present we have two sets of evidence that help support this concept, namely the observation that the increase in conductivity of the protein-complexes is roughly mirrored by an increase in the free radical concentration, and the fact that the magnitudes of the dielectric dispersion and free radical concentration remain independent of temperature to within experimental accuracy.

If the assumption is made that the number n_s of free spins observed from the e.s.r. measurments does in fact represent the number of separated electronic charges then a value for the microscopic mobility μ_o can be estimated using equations (1) and (5) together with the standard conductivity equation

$$\sigma = 1/2(n_s q \mu_m) = 1/2(n_s q C \mu_o)\exp(-\Delta kT) \qquad (7)$$

The factor 1/2 is included because the charge accepted by the (Schiff base linked) methylglyoxal is assumed to be relatively immobile. Since n_s has been found to be independent of temperature the conductivity activation energy ΔE has been interpreted as representing the mobility activation energy in equation (5). From the observed values for n_s and ΔE, and knowing that the value for C should be greater than unity, we can estimate for the BSA, casein and lysozyme complexes that $\mu_o \simeq 10^{-6}$ $m^2/Vsec$, whereas for the collagen complex $\mu_o > 10^{-2}$ $m^2/Vsec$. By equating N in equation (3) with $1/2n_s$, then from an analysis of the hopping charge carrier model[13,18] used to describe the dielectric dispersions the average distance travelled by the charges surmounting the potential barriers is of the order

6 to 8 nm for the BSA, casein and lysozyme complexes, and about 75 nm for the collagen complex. By comparing these distances with the complete charge separation distance of 18 nm derived from equation (6), and considering the estimations of the microscopic mobility, then of the protein complexes studied the collagen complex may be the only one for which charge transport in bands of extended states can occur. This may arise from the fact that the large molecular weight of collagen allows for a sufficiently large number of atomic interactions to occur and so form well-defined energy bands. Although charge transport for the other protein complexes most probably involves a hopping or phonon-assisted tunneling process, a mean "hop distance" of the order of 6 to 8 nm still represents a significant degree of charge mobility at the molecular level.

Although these measurements represent just the first few steps in what we expect will prove to be an exciting and fruitful journey, we believe they provide strong evidence for the concept that proteins can take part in charge-transfer interactions which in turn results in the appearance of mobile electronic charges within the protein structure. Such an effect will lead to a whole range of submolecular electronic subtleties whose relevance up until now has largely been overlooked by the biological sciences.

Methylglyoxal - Ascorbic Acid Compound

Finally, we wish to mention a recent development which helps convince us that the charge-transfer concepts we have described here are of biological and medical importance

Following the preliminary spectroscopic and e.s.r. studies with Jane McLaughlin and P. R. C. Gascoyne it was concluded (ref 4, pp 46 -59) that the ascorbic acid molecule or one of its metabolic derivatives could play a role in charge-transfer processes with proteins by boosting the electron accepting action of aldehydes, such as methylglyoxal, and in doing so assist the transfer of electrons to the final electron acceptor, oxygen. The idea therefore arose that a compound of methylglyoxal and ascorbic acid might have significant biological activity. Recently such a novel compound has been synthesized in the laboratory of G. Fodor, and the reaction is thought to proceed via the initial formation of the 3-hemiacetal between the aldehydic group of methylglyoxal and the 3-OH group of ascorbic acid. Recent quantum chemical calculations[22], assisted by space-filling molecular models,have demonstrated that molecular conformations are possible in which charge can be transferred from the nitrogen atom of a neighbouring peptide group to the Schiff base formed between the ε-amino group of a lysine side-chain and the ascorbic acid methylglyoxal acetal. Furthermore, these calculations indicate that the charge transfer reactions will be stronger for the system ascorbic acid-Schiff base relative to the Schiff base alone. This new

compound has been found to produce interesting physiological and anti-tumour activity. These studies will be published by the various investigators concerned, and our only purpose in mentioning them here is that we believe that such an interesting compound is most unlikely to have been developed by "pure chance". Rather it demonstrates the basic validity of the type of approach described here, and can be taken as a portent of the future achievements that will develop from just this one branch of bioelectrochemistry.

REFERENCES

1. A. Szent-Györgyi, "Introduction to a Submolecular Biology", Academic, New York (1969)
2. A. Szent-Györgyi, "The Living State", Academic, New York (1972)
3. A. Szent-Györgyi, "Electronic Biology and Cancer", Marcel Dekker, New York (1976)
4. A. Szent-Györgyi, "The Living State and Cancer", Marcel Dekker, New York (1978)
5. R. Pethig, "Dielectric and Electronic Properties of Biological Materials", Wiley, Chicester (1979)
6. D. L. Worcester, Proc. Natl. Acad. Sci. USA, 75, 5475 (1978)
7. A. Szent-Györgyi, Nature, 148 157 (1941)
8. G. Foder, R. Mujumdar and A. Szent-Györgyi, Proc. Natl. Acad. Sci. USA, 75, 4317 (1978)
9. H. D. Dakin and H. W. Dudley, J. Biol. Chem. 14, 155 (1913)
10. C. Neuberg, Biochem. Z. 49, 202 (1913)
11. P. Otto, J. Ladik, K. Laki and A. Szent-Györgyi, Proc. Natl. Acad. Sci. USA, 75, 3548 (1978)
12. R. Pethig, Int. J. Quantum Chem: Quantum Biol. Symp. 5, 159, (1978)
13. S. Bone and R. Pethig, "Submolecular Biology and Cancer", Ciba Foundation Symposium 67, (new series), Elsevier, Holland, pp 83-105 (1979)
14. R. Pethig and A. Szent-Györgyi, Proc. Natl. Acad. Sci. USA, 74, 226 (1977)
15. C. C. Liang, Trans. Faraday Soc. 65. 3369 (1969)
16. J. Eden, P. R. C. Gascoyne and R. Pethig, J. Chem. Soc. Faraday I, 75 (1979). In print.
17. P. Carnochan and R. Pethig, J. Chem. Soc. Faraday I, 72, 2355 (1976)
18. T. J. Lewis, Int. J. Quantum Chem: Quantum Biol. Symp. 5. 149 (1978)
19. P. R. C. Gascoyne and R. Pethig, J. Chem. Soc. Faraday I, 73, 171 (1977)
20. R. Pethig, J. Biol. Phys. 1, 193 (1973)
21. R. Pethig and R. B. South, IEEE Trans. Instr. Meas. IM. 23, 406 (1975)
22. P. Otto, J. Ladik and A. Szent-Györgyi, Proc. Natl. Acad. Sci. USA, 76 (1979). In print.